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CRISPR/Cas in Arabidopsis: overcoming challenges to accelerate improvements in crop photosynthetic efficiencies

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The rapid and widespread adoption of CRISPR/Cas technologies has allowed genetic editing in plants to enter a revolutionary new era. In this mini-review we highlight the current CRISPR/Cas tools available in plants and the use of *Arabidopsis thaliana* as a model to guide future improvements in crop yields, such as enhancing photosynthetic potential. We also outline the current socio-political landscape for CRISPR/Cas research and highlight the growing need for governments to better facilitate research into plant genetic editing technologies.

Introduction

Genetic editing via CRISPR/Cas has been used by plant biologists for a range of purposes, from generating novel mutants for fundamental biological studies to improving crop plant performance and enhancing crop yields (recently reviewed in Scheben and Edwards 2018). Although CRISPR/Cas is now a well-known tool, its first use in plants and other eukaryotes was reported only five years ago (Nekrasov et al. 2013). Thus, CRISPR/Cas is still a relatively immature technology and new findings and applications continue to emerge, promising to further enhance our capabilities for precise genetic

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editing in plants. The purpose of this short review is to provide an update on the current successes and challenges of CRISPR/Cas in plant research with a key focus on *Arabidopsis thaliana* (Arabidopsis), the most well studied model plant species. We will discuss the continued usefulness of Arabidopsis as a model for guiding genetic editing strategies, in particular, for improving photosynthetic efficiencies and crop yields. This review is also particularly timely, given the recent opposing rulings in the US and EU on the status of genetically edited plants. Therefore, we will also briefly consider the political, social and commercial aspects of the CRISPR/Cas research landscape.

CRISPR/Cas gene editing in action

The CRISPR/Cas gene editing system is a repurposed domestication of the class II CRISPR (clustered regularly interspaced short palindromic repeats) interference mechanism from the adaptive immune response of prokaryotes (Cong et al. 2013, Nekrasov et al. 2013). CRISPR/Cas relies on the interaction of a CRISPR-associated endonuclease (Cas) enzyme with a synthetic guide RNA (gRNA) designed to target and induce cleavage at specific DNA or RNA sites (for a detailed mechanistic review see Jiang and Doudna 2017). In brief, sequence-specificity is achieved by a short region (19-22 nt) in the gRNA that is complementary to the host target sequence and next to a 3-6 nt protospacer adjacent motif (PAM) sequence (Table 1; Jinek et al. 2012). Off-target mutations can occur at undesired sites that have mismatches distal to the PAM (Zhang et al. 2018). However, several bioinformatic tools are now available to predict off-target activity based on the gRNA(s) and Cas used, which can subsequently be screened for during analysis (reviewed in Zischewski et al. 2017). As such, the majority of studies in plants report a low frequency of mutation at off-target sites (Xie et al. 2014, Jacobs et al. 2015). Class II Cas (comprising types II, V and VI Cas) are currently the most attractive targets to domesticate for genetic editing as they can perform several tasks in one, including formation of a ribonucleoprotein complex with gRNAs and the processing of those gRNAs, as well as

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recognition of the PAM site within the host target sequence (Shmakov et al. 2016). Host sequence disruption is achieved via the error-prone repair of double-stranded breaks (DSBs) by the native eukaryotic non-homologous end joining pathway (NHEJ), which typically induces small insertions or deletions (indels) at the DSB site. More precise genomic deletions or insertions can be generated through homology-directed repair (HDR) of DSBs with a template or ‘donor’ sequence (Li et al. 2013, Knoll et al. 2014).

The type II Cas9 from *Streptococcus pyogenes* (SpCas9) was the first reported Cas isoform to be domesticated (Jinek et al. 2012). Although several others Cas9 variants have since been studied (Table 1), SpCas9 is still the most commonly used to generate loss of function mutations in plants (Nekrasov et al. 2013, Sánchez-León et al. 2018). The two nuclease domains of Cas9, RuvC and HnH, induce a blunt-ended DSB, three base pairs upstream of the PAM sequence (Jiang and Doudna 2017). More recently, additional structurally and functionally distinct class II Cas homologs have been identified with the capacity to cleave DNA or RNA (Shmakov et al. 2016). For example, the type V Cas12a (previously known as Cpf1) is functionally similar to Cas9, but generates a staggered DSB (i.e. a four-nucleotide overhang) upstream of the PAM and outside of the gRNA sequence using a single RuvC nuclease domain (Zetsche et al. 2015). The PAM sequences of Cas12a are well-suited for targeting AT-rich genomic regions, such as promoters. Cas12a also has RNase activity and can process a sequential string of gRNAs from a single promoter to more easily facilitate multiple gene targeting (known as multiplexing; Wang et al. 2017a). The type VI Cas13a (previously known as C2c2) targets and cleaves RNA using the ribonuclease domain HEPN (Abudayyeh et al. 2018). CRISPR/Cas-based RNA targeting could have important applications in functional RNA studies and generating resistance to common single-stranded RNA plant viruses (e.g. Cauliflower mosaic virus; Aman et al. 2018). Modified variants of Cas have also expanded the capabilities of CRISPR/Cas. For example, catalytically inactivated Cas (dCas) retains the capacity for target binding and can be used to

regulate gene expression through transcriptional interference (a process called CRISPRi by Qi et al. 2013). dCas fused to transcriptional repression or activation domains can be used in plants for modulation of gene expression (Tang et al. 2017, Lowder et al. 2018) and epigenetic modification (Gallego-Bartolomé et al. 2018). More recently, the potential applications for targeted mutagenesis have been further developed by fusing dCas9 with a cysteine or adenine deaminase domain for precise base editing of C/G to T/A or T/A to C/G, respectively (Eid et al. 2018, Li et al. 2018). Base editing could be applied to engineer alternative variants of enzymes and/or regulatory sequences in cases requiring a single nucleotide change, thus removing the need to supply a donor template for HDR-based approaches.

Arabidopsis – leading the way or playing catch up?

CRISPR/Cas has been central to a recent surge in genetic editing studies in a variety of crop species. A key aim is to engineer desirable agronomic traits, such as abiotic stress resilience and pathogen resistance, and develop transgene-free edited plant lines (Scheben and Edwards 2018). Two outstanding examples are the generation of low-gluten wheat lines (Sánchez-León et al. 2018) and the domestication of wild tomato (Zsögön et al. 2018). Improvements in Cas and gRNA(s) delivery methods, high frequencies of editing in transformants and better tissue culture regeneration methods have accelerated the development of non-model, polyploid plants for functional and applied reverse genetic research (e.g. Li et al. 2017, Lin et al. 2018). As such, CRISPR/Cas will likely continue to drive efforts to expand the availability of reference genomes in different plant species.

In contrast, CRISPR/Cas work in the model species *Arabidopsis* has been highlighted by several challenges associated with localisation of Cas expression, relatively low transformation efficiencies and issues with heritability (Feng et al. 2014, Mao et al. 2016). One drawback has arisen from a key advantage: *Arabidopsis* can be transformed at high efficiencies by the floral dip method, which is

more rapid than tissue culture approaches required for most other plant species (Fig. 1). Although floral dipping has significant benefits over tissue culture approaches, the frequency of heterozygous, homozygous and bi-allelic CRISPR/Cas induced mutations initially reported in T1 Arabidopsis lines has been low (Table 2), with 1-bp indels and chimeric mutations in somatic cells accounting for the majority of mutation types (Feng et al. 2014). This issue has been linked to the use of common, strong promoters to drive Cas expression [e.g. the Cauliflower mosaic virus promoter (CaMV35S)], which have been shown to have a low activity in germ-line cells or at the one-cell stage of embryogenesis (Hyun et al. 2015, Wang et al. 2015). In contrast, plants regenerated through tissue culture can arise through embryogenesis from a single somatic cell where such promoters are highly active. Tissue culture-based studies using CaMV35S to drive Cas expression have reported homozygous mutations associated with NHEJ in T0 lines for a variety of species, including the woody plant *Populus tomentosa* (Fan et al. 2015).

Several crop species have also made significant progress in HDR-based editing (Butler et al. 2016, Sun et al. 2016). However, the frequency of HDR events varies considerably between plant species, with low efficiencies initially being reported in Arabidopsis. For example, Li et al. (2013) compared the delivery of a double stranded DNA (dsDNA) donor template (via PEG-mediated transformation) to *Nicotiana tabacum* (tobacco) and Arabidopsis protoplasts. HDR-mediated integration of the donor template was unsuccessful in Arabidopsis, whereas an integration frequency of 9% was reported for tobacco. Similarly, Schiml et al. (2014) reported only a low frequency of donor template integration (ca. 0.1%) in Arabidopsis. *Agrobacterium*-mediated transformation of geminivirus-based vectors can help to increase the abundance of the donor template, and has yielded improved donor integration frequencies in tomato and rice (6 and 8.5%, respectively; ermák et al. 2015, Wang et al. 2017b). Viral-mediated genome editing was recently reported in Arabidopsis (Ali et al. 2018), although it has not yet been applied for HDR-mediated strategies.

Return of the King: increasing the efficiencies of CRISPR/Cas editing in Arabidopsis

Numerous studies have now shown that using germline-specific promoters to express Cas can significantly improve the frequency and heritability of mutations in Arabidopsis (Table 2; Hyun et al. 2015, Wang et al. 2015, Yan et al. 2015, Mao et al. 2016). Germline-specific promoters can increase the frequencies of homozygous mutations in T1 plants and lower the rate of chimerism compared to non-germline specific promoters such as CaMV35S (Wang et al. 2015, Yan et al. 2015, Mao et al. 2016). This has reduced the sample size needed for screening and the requirement for multi-generational analyses of mutations. To date, 13 germline-specific promoters have been reported, with heritable mutation rates of up to 17% in the T1 generation with EC1.1/EC1.2 (Hyun et al. 2015, Wang et al. 2015, Yan et al. 2015, Eid et al. 2016, Mao et al. 2016, Osakabe et al. 2016). Recently, a robust protocol for HDR-based editing using a germ-line specific promoter (DD45) to drive Cas9 expression was reported in Arabidopsis with a knock-in efficiency of 16-55% observed in the T2 generation (Miki et al. 2018). Additionally, replacing the constitutive promoter PcUbi4-2 with EC1.1 improved the rate of HDR from 1 to 6% (Wolter et al. 2018). Together, these results indicate that the timely expression of Cas in germ cells or during early embryogenesis is a critical factor for HDR-directed editing in Arabidopsis.

Based on recent work, there are many other opportunities to enhance gene editing efficiencies in Arabidopsis. Ordon et al. (2018) reported that improvements in vector design coupled with a paired gRNAs approach resulted in high frequencies (1.6%) for a 70 kb deletion using a constitutive ubiquitin promoter (Ordon et al. 2018). Better transformant screening strategies, improvements in gRNA expression and a more detailed understanding of the variability in gRNA efficiencies should help to further increase the detection and frequency of heritable mutations (Ordon et al. 2018, Wu et al. 2018). Increases in mutation frequencies can also be achieved by subjecting plants to periodic heat

stress that favours the activity of currently used Cas enzymes (LeBlanc et al. 2017). Future work could focus on identifying Cas variants that have maximal activity at temperatures used for plant growth.

From labs to fields: how Arabidopsis can guide improvements in crop photosynthesis

CRISPR/Cas has successfully improved agronomic traits in a variety of crops. Although the transformation and selection of some crops is now routinely achieved (e.g. in rice and wheat), generating transgenic lines remains a labour-intensive process for many species. Arabidopsis has historically been a powerful model species to study gene function and regulation. Due to recent advances in CRISPR/Cas editing, Arabidopsis remains well-positioned as a rapid and convenient tool to screen crop improvement strategies for complex traits that involve multiple genes and/or gene families. Increasing the efficiency of photosynthesis to improve productivity is one key example. Many approaches have been suggested, which include (and sometimes combine) enhancing the capacity for light capture, reducing photorespiration, and increasing flux through the Calvin cycle (Zhu et al. 2007, Ort et al. 2015, Rae et al. 2017 South et al. 2018). Recent lab- and field-based studies using transgenic plants have now shown that enhancing photosynthesis is a transformative strategy that can increase yields (Simkin et al. 2015, Kromdijk et al. 2016, Driever et al. 2017, Lopez-Calcano et al. 2018). The multiplexing approaches achievable with CRISPR/Cas can be screened more rapidly in Arabidopsis to enable the progression of more complex strategies, as typically numerous genes are involved that require appropriate regulation. For example, there are 38 enzymes directly involved in photosynthetic carbon assimilation in C3 plants [e.g. the Calvin-Benson cycle (CB) and photorespiratory pathway] and multiple chaperones and regulatory components encoded by several gene families (Zhu et al. 2007).

Models to optimise photosynthetic carbon metabolism indicate that modifying the activity of photorespiratory enzymes, Rubisco, and increasing the activity of other CB enzymes can increase photosynthesis (Zhu et al. 2007). For the latter, overexpression of a single enzyme, Sedoheptulose-1, 5 biphosphatase (SBPase), can increase photosynthetic rates in model species and crops, including wheat and tomato (Lefebvre et al. 2005, Ding et al. 2016, Driever et al. 2017). This approach has been developed further by overexpressing SBPase with an additional CB enzyme (Fructose 1,6-bisphosphate aldolase) in Arabidopsis and tobacco (Simkin et al. 2017, Simkin et al. 2015, respectively). These examples represent significant progression towards increasing photosynthesis by manipulating the activity of multiple enzymes simultaneously. CRISPR/Cas-mediated HDR or NHEJ could allow similar strategies to be commercially applied to crops, for example, by modulating the activity of native enzymes and/or promoter-driven transcription. However, progress in understanding the regulation and diversity of other pathways related to photosynthesis (e.g. photorespiration, C4 and crassulacean acid metabolism (CAM) pathways, and pyrenoid-based CO₂-concentrating mechanisms) would also be accelerated by the approaches discussed. Therefore, in the immediate future, CRISPR/Cas will likely be applied to functional studies in model species to elucidate the regulation and activity of new targets for manipulation.

A key challenge in genetic engineering has been the manipulation of enzymes that are represented by, or regulated by gene families. For example, the small subunit of Rubisco (rbcS) has multiple isoforms (four in Arabidopsis and up to 22 in other species), while assembly with the plastid encoded Rubisco large subunit (rbcL) requires at least five species-specific chaperones in plants (Spreitzer 2003, Aigner et al. 2017). CRISPR/Cas-based approaches could overcome challenges associated with engineering gene families, including Rubisco. Engineering the catalytic site, which is located on the rbcL, is mostly limited to species amenable to routine plastid transformation, such as tobacco. However, the nuclear-encoded rbcS is also known to influence Rubisco catalysis (Spreitzer 2003,

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Atkinson et al. 2017). Recently, a new group of specialised rbcS, called rbcS-T, have been identified that are expressed exclusively in plant organs with specialised metabolism (e.g. trichomes; Laterre et al. 2017, Pottier et al. 2018). Endogenous rbcS-T isoforms likely maintain chaperone specificity but can alter the catalytic properties of Rubisco (Morita et al. 2014, Laterre et al. 2017). Thus, replacing rbcS expressed in mesophyll cells with an rbcS-T isoform could improve the efficiency of leaf CO₂ assimilation. Alternatively, as rbcS-T isoforms are not found in all species (Pottier et al. 2018) it may be desirable to express isoforms that are significantly divergent from the native family. Although the chaperones involved in Rubisco assembly appear to be highly species-specific (Aigner et al. 2018), little is known of the mechanisms underlying chaperone specificity in planta. Replacing and/or modifying endogenous chaperones via CRISPR/Cas-mediated HDR or base-editing could accelerate fundamental studies underpinning chaperone involvement in rbcS and rbcL assembly.

CRISPR/Cas based approaches offer the potential to improve existing strategies to increase photosynthesis, including enzyme overexpression (as HDR-mediated strategies improve), and to overcome key challenges, such as manipulating Rubisco. With improved plastid transformation protocols in different species (Yu et al. 2017), it may soon be possible to reliably engineer both plastid and nuclear expressed photosynthetic enzymes and/or associated regulatory proteins (Avila et al. 2016). Thus, Arabidopsis remains a critically important platform to rapidly test novel strategies in planta and examine the impact of photosynthetic efficiency and productivity before undertaking time-consuming translational studies.

When politics triumphs over science: the possibility of gene-edited food on your plate

Although CRISPR/Cas has resulted in a significant increase in agri-tech investment, social acceptance and discrepancies surrounding the regulation of gene editing technologies still hinders basic research and commercialisation in most countries (Smart et al. 2016, Brinegar et al. 2017). The adoption of

genetically modified (GM) crops by farmers is increasing globally (Parisi et al. 2016), but opposition by consumers to GM food is still highly prevalent (Blancke et al. 2015). Whilst the public perception of products engineered by gene editing is unclear, the regulatory status of organisms produced by these methods will play a central role in social acceptance. The US Department of Agriculture (USDA) have recently announced that plants produced by gene editing, which could be made by traditional breeding techniques, will not be subject to genetically modified organism (GMO) regulations (USDA 2018). This ruling includes plants with gene deletions of any size, single base pair substitutions and *cis*-genic plants. The announcement is a boon for the US agri-tech industry and will encourage biotech companies to invest in plant genetic editing research without the risk of facing costly regulatory processes (Smart et al. 2017). Products already planned for the market include sweeter strawberries with a longer shelf life (Monsanto) and drought-resistant maize lines (DuPont Pioneer).

In contrast, a recent landmark ruling by the EU Court of Justice has applied the same stringent regulations for conventional GMOs to genetically-edited plants (for an excellent review of the regulatory framework see Agapito-Tenfen et al. 2018). In the EU, a GMO is defined as “an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.” This definition covers both the process of genetic modification and the final product, so plants produced through lab-based technologies, including *cis*-genic plants that contain genes from sexually compatible species must be labelled as GMO (Agapito-Tenfen et al. 2018). The ruling came as a disappointment to EU-based scientists, as the negative effect of GMO legislation, that has hindered research for the past 15 years, will continue to impact new gene editing technologies and commercial uptake in the EU (Callaway 2018). In the future, the European commission may seek to overturn the court’s ruling. Resolving the current international discrepancies between gene editing regulations will help global efforts to ameliorate impending food security concerns.

Author contributions

P.K. and S.D. had a primary role in writing the manuscript, including drawing figures and tables. A.J.M. read and corrected the manuscript.

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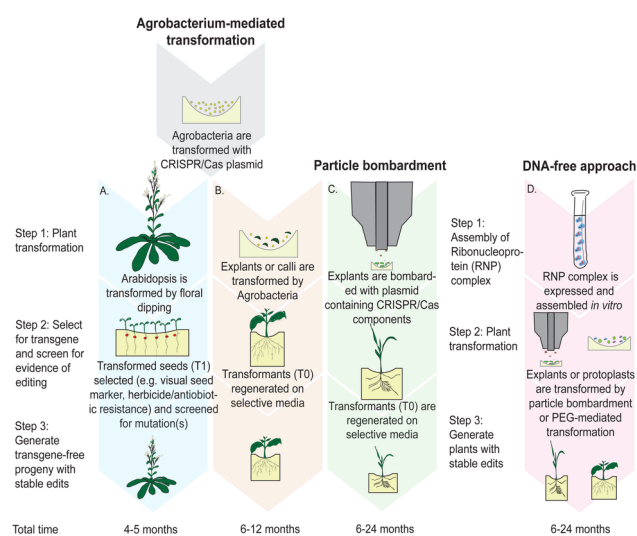
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Figure legend

Fig. 1. A comparison of workflows and timescales to obtain genetically-edited transgene free plants using CRISPR/Cas. (A) Floral dipping of Arabidopsis. (B) Agrobacterium-mediated transformation of other dicot species (e.g. tobacco) and regeneration of explants or calli by tissue culture. (C)

Transformation of monocot species (e.g. wheat) by particle bombardment and regeneration by tissue culture. (D) Intracellular delivery of a ribonucleoprotein (RNP) gRNA-Cas complex and regeneration by tissue culture. Transformants are screened for editing events by PCR and sequencing or commonly used mutation assays (e.g. Surveyor assay, T7 endonuclease assay). The period of time until transgene-free progeny are identified is species-dependent.



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Table 1. List of Cas variants used in some of the plant studies referenced in this review. Cas targets different PAM sequences, thus expanding the available genomic sites for gene editing. The RNA-targeting Cas13a variants used in plants so far have been PAM-independent. PAM sequence abbreviations: N: any nucleotide, V: A, C or G; R: A or G; Y: C or T; M: A or C

Cas variant	Source species	Size (a.a.)	PAM sequence(s)	Plant species used	References
SpCas9	<i>Streptococcus pyogenes</i>	1368	NGG	Species referenced in this review include <i>Arabidopsis</i> , <i>Nicotiana benthamiana</i> , <i>Zea mays</i> (maize), <i>Hordeum vulgare</i> (barley), <i>Brassica oleracea</i> (broccoli), <i>Bambusa oldhamii</i> (bamboo), <i>Setaria italic</i> (millet), <i>Brassica napus</i> (rapeseed) <i>Oryza sativa</i> (rice), <i>Solanum pimpinellifolium</i> (wild tomato)	Nekrasov et al. 2013, Xing et al. 2014, Lawrenson et al. 2015, Lin et al. 2018, Zsögön et al. 2018
StCas9	<i>Streptococcus thermophilus</i>	1122	NNAGAA, NNGGAA	<i>Arabidopsis</i>	Steinert et al. 2015
SaCas9	<i>Staphylococcus aureus</i>	1053	NNGGGT, NNGAA	<i>Arabidopsis</i>	Steinert et al. 2015, Wolter et al. 2018
LbCas12a	<i>Lachnospiraceae bacterium</i> ND2006	1228	TTTV	<i>O. sativa</i> , <i>Arabidopsis</i> , <i>Glycine max</i> (soybean), <i>Nicotiana attenuata</i> (wild tobacco)	Kim et al. 2017, Tang et al. 2017
AsCas12a	<i>Acidaminococcus</i> sp. BV3L6	1307	TTTV	<i>O. sativa</i> , <i>Arabidopsis</i> , <i>G. max</i> , <i>N. attenuata</i>	Kim et al. 2017, Tang et al. 2017
FnCas12a	<i>Francisella novicida</i>	1300	TTV	<i>O. sativa</i> , <i>N. benthamiana</i>	Endo et al. 2016
LwaCas13a	<i>Leptotrichia wadei</i>	1212	PAM-independent	<i>O. sativa</i>	Abudayyeh et al. 2018
LshCas13a	<i>Leptotrichia shahii</i>	1389	PAM-independent	<i>N. benthamiana</i>	Aman et al. 2018

Table 2. Promoters used to drive Cas expression in Arabidopsis. The frequencies of heritable mutations in T1 and T2 generations are indicated.

Promoter	Terminator	Target gene(s)	Targeted organ expression	Heritable mutation in T1 (%)	Heritable mutation in T2 (%)	Reference
AtEC1.1; AtEC1.2; EC1.1/1.2	PsRbcSE9	<i>ETC2, TRY, CPC, CHLI1/2</i>	Egg cells, embryo	1.8; 8.3; 17.0	N/A	Wang et al. 2015
AtYAO	Nopaline synthase (Nos) from <i>A. tumefaciens</i>	<i>BRI1</i>	Embryo sac, embryo, endosperm and pollen	6.7	66	Yan et al. 2015
AtINCURVATA2	Nos	<i>FT, SPLA4</i>	Endosperm and embryo	13.0	N/A	Hyun et al. 2015
AtSPOROCTELESS (SPL)	AtSPL	<i>AP1, TT4</i>	Early microsporocytes and megasporocytes	0	70	Mao et al. 2016
AtDD45	Nos	<i>GL2</i>	Zygotes and early embryo	5.6	38	Mao et al. 2016
SILAT52	Nos	<i>GL2</i>	Pollen	0	39	Mao et al. 2016
AtRPS5A	AtHSP	<i>Adh1</i>	Constitutive	81	N/A	Tsutsui and Higashiyama 2017
AtUBQ10	PsRbcSE9	<i>At3g04220</i>	Constitutive	74	N/A	Wu et al. 2018
PcUBQ4-2	Nos	<i>DM2C</i>	Constitutive	10	N/A	Ordon et al. 2017
CaMV35S	Nos	<i>BRI1, JAZ1, GAI, CHLI, TT4</i>	Constitutive	0	22	Feng et al. 2014